QP501.B57

STIC-ILL

From: Shibuya, Mark
Sent: Tuesday, August 29, 2000 4:36 PM
To: STIC-ILL
Subject: Reference request for 09/403,539 Dean et al. alimentary

Please send me the following references:

Nucleic acids research (ENGLAND) Apr 1 1998, 26 (7) p1567-75, ISSN 0305-1048

Journal of Pharmacology and Experimental Ther eutics 286 (1):p 419-428 July, 1998, ISSN: 0022-3565

Bioorganic & Medicinal Chemistry Letters 7 (1 1791-1796, 1997, ISSN: 0960-894X

Thank you,

Mark Shibuya
Examiner, AU 1635
308-9355
CM1, 11D11

2e-

√

ADONIS - Electronic Journal Services

Requested by

Adonis

Article title	Synthesis and properties of hammerhead ribozymes stabilized against nucleases by different 2'-modifications: Methoxyethoxy-, fluoro- and amino groups
Article identifier	0960894X98017993
Authors	Scherr_M Klebba_C Haner_R Ganser_A Engels_J_W
Journal title	Bioorganic and Medicinal Chemistry Letters
ISSN	0960-894X
Publisher	Pergamon
Year of publication	1997
Volume	7
Issue	13
Supplement	0
Page range	1791-1796
Number of pages	6
User name	Adonis
Cost centre	
PCC	$20.00
Date and time	Wednesday, August 30, 2000 8:09:28 PM

Copyright © 1991-1999 ADONIS and/or licensors.

The use of this system and its contents is restricted to the terms and conditions laid down in the Journal Delivery and User Agreement. Whilst the information contained on each CD-ROM has been obtained from sources believed to be reliable, no liability shall attach to ADONIS or the publisher in respect of any of its contents or in respect of any use of the system.

18069302

Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 13, pp. 1791-1796, 1997
© 1997 Elsevier Science Ltd
All rights reserved. Printed in Great Britain
0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00313-2

Synthesis and properties of hammerhead rib zymes stabilized against nucleases by different 2'-modifications: methoxyethoxy-, fluoro- and amino groups

Michaela Scherr, Christian Klebba, Robert Häner[a], Arnold Ganser[b] and Joachim W. Engels*

Institut für Organische Chemie, Johann Wolfgang Goethe-Universität, Marie-Curie-Str. 11, D-60439 Frankfurt am Main, Germany
[a] *Novartis Pharma AG, Core Technology Area, CH-4002 Basel, Switzerland*
[b] *Medizinische Hochschule Hannover, Abt. Hämatologie und Onkologie, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany*

Abstract: 2'-modified ribonucleosides (2'-methoxyethoxy- O, 2'-fluoro-2'-deoxypyrimidines O and 2'-amino-2'-deoxyuridines □) were introduced into hammerhead ribozymes. Kinetics and stability assays were performed. The lipophilicity characteristics of these 2'-modifications were investigated measuring partition coefficients and HPLC retention times. © 1997 Elsevier Science Ltd.

Ribozymes have potential as therapeutic entities for the treatment of e.g. cancer[1-3] or HIV[4]. For exogenous delivery into cells they have to be stabilized against nucleolytic degradation without loss of their catalytic efficiency.

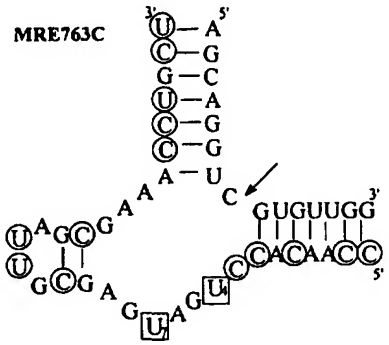
Hammerhead ribozymes are catalytic RNA molecules which recognize substrates containing NUH base triplets (N can be any base; H can be A, C, or U) and cleave a phosphodiester bond on the 3'-side of H in *trans* position specifically in the presence of divalent metal ions (e.g. Mg^{2+})[5-7]. The 2'-position has to be modified as the 2'-hydroxyl group is involved in the degradation of the ribozyme. We examined the influence of chemical modifications such as 2'-methoxyethoxy- and 2'-fluoro-2'-deoxypyrimidine combined with 2'-amino-2'-deoxyuridines in U_4 and U_7 positions on the catalytic activity and stability of hammerhead ribozymes targeted against N-*ras* transcripts containing point mutations in codon 13. Previous studies[8,9] showed that replacement of all pyrimidine nucleotides by their 2'-fluoro derivatives in the hammerhead domain except U_4 and U_7 positions demonstrated high cleavage activity. But due to the remaining unmodified ribose units they were degraded in cell culture supernatants containing 10 % FCS in less than 30 minutes. The substitution of the U_4 and U_7 positions in the catalytic core with 2'-amino-2'-deoxyuridine led only to a slight loss of catalytic activity compared to the unmodified one. But this structural change resulted in a remarkable stability against RNases present in the cell culture containing 10% FCS as described by Heidenreich et al[10]. Beigelman et al. obtained also improved cellular stability with 2'-modifications such as 2'-C-allyl-, 2'-O-methyl- and 2'-amino groups[11].

When we replaced the 2'-hydroxy group by methoxyethoxy in all pyrimidine nucleotides O and in the U_4 and U_7 positions □ by 2'-amino-2'-deoxy groups in the ribozyme M3 (Figure 1), half of the cleavage efficiency

* Fax: (49) (69) 7982-9148; E-mail: engels@ews1.org.chemie.uni-frankfurt.de

toward the short synthetic oligonucleotides under 'steady-state' conditions was retained compared to the unmodified ribozyme (Table 1).

Figure 1. Design of the modified hammerhead ribozyme



For the 2'-fluoro-2'-deoxypyrimidines O and the 2'amino-2'-deoxyuridines □ modified ribozyme M1 (4- and 7-positions) only 40% of the wildtype activity was preserved. Total substitution, including U_4- and U_7-positions, of the 2'-hydroxy group by 2'-methoxyethoxy pyrimidine in the ribozyme M5 resulted in a complete loss of activity (k_{cat}/K_M = $5.2 \cdot 10^{-2}$ $min^{-1} \cdot nM^{-1}$ for the unmodified ribozyme to $2.6 \cdot 10^{-4}$ for the modified ribozyme M5).

Table 1. K_M and k_{cat} values of ribozymes with short synthetic substrates

Ribozyme MRE763C	modification	k_{cat} [min^{-1}]	K_M [nM]	k_{cat}/K_M [$min^{-1} \cdot nM^{-1}$]	k_{cat}/K_M (relative)
	unmodified	3.760	72	0.05100	1
M1	**FU, FC, U_4U_7-NH_2**	0.300	16	0.01900	0.380
M3	**MOE-T, MOE-C, U_4U_7-NH_2**	0.530	22	0.02400	0.470
M5	**MOE-T, MOE-C, U_4U_7-MOE**	0.027	105	0.00026	0.005
M7	**S, MOE-T, MOE-C, U_4U_7-NH_2**	0.220	18	0.01200	0.230

The k_{cat}- and K_M-values were determined under 'steady-state' conditions. Terminal phosphorothioate groups are indicated by an S. The abbreviations MOE-T and MOE-C are 2'-methoxyethoxy-2'-deoxythymidine and 2'-methoxyethoxy-5-methylcytidine.

For further investigations, in order to optimize mRNA-cleavage, the *in vitro* transcribed N-*ras* RNA, a 849 nucleotide long transcript[9], served as substrate. The catalytic efficiency was examined under 'single-turnover' conditions (Table 2). Modifications such as 2'-methoxyethoxy-2'-deoxypyrimidine and 2'-amino-2'-deoxyuridines in the ribozyme M3 (U4- and U7-positions) led only to a slight loss of activity. The k_{react}/K_M-value is 2370 $s^{-1} \cdot M^{-1}$ compared to 3752 $s^{-1} \cdot M^{-1}$ of the unmodified ribozyme. The ribozyme M1 containing 2'-amino-2'-deoxyuridines at positions U_4 and U_7 and 2'-fluoro-2'-deoxy groups posseses nearly equal k_{react}/K_M-

values compared to the above mentioned 2'-methoxyethoxy-2'-deoxypyrimidine and 2'-amino-2'-deoxyuridine containing ribozyme M3.

Table 2. k_{react}/K_M-values of different modified ribozymes with *in vitro* transcribed N-*ras* RNA

Ribozyme MRE763C	modification	k_{react} [10^{-6} s^{-1}]	K_M [nM]	k_{react}/K_M [s^{-1} M^{-1}]	k_{react}/K_M (relative)
	unmodified	266	71	3752	1
M1	**FU, FC, U_4U_7-NH_2**	173	71	2437	0.650
M2	**MOE-T, MOE-C, U_4U_7-OH**	147	51	2882	0.770
M3	**MOE-T, MOE-C, U_4U_7-NH_2**	173	73	2370	0.630
M4	**MOE-T, MOE-C, U_4U_7-F**	39	135	288	0.077
M5	**MOE-T, MOE-C, U_4U_7-MOE**	nd	nd	nd	nd
M6	**S, MOE-T, MOE-C, U_4U_7-F**	nd	nd	nd	nd
M7	**S, MOE-T, MOE-C, U_4U_7-NH_2**	27	190	142	0.038

The k_{react}- and K_M-values were determined under 'single-turnover' conditions [nd = not detectable].

In Table 3 the stability data of such modified ribozymes are listed. It is remarkable that the 2'-methoxyethoxy modified ribozymes are much more stable in cell culture supernatants supplemented with 10% FCS than the 2'-fluoro-2'-deoxy compound M1. This could have important implications for cell experiments.

Table 3. Stability of chemically modified ribozymes in cell culture supernatant supplemented with 10% FCS

Ribozyme MRE763C	modification	cell supernatants (half-life times)
	unmodified	0.5 min
M1	**FU, FC, U_4U_7-NH_2**	50 h
M2	**MOE-T, MOE-C, U_4U_7-OH**	30.0 min
M3	**MOE-T, MOE-C, U_4U_7-NH_2**	80 h
M4	**MOE-T, MOE-C, U_4U_7-F**	80 h
M5	**MOE-T, MOE-C, U_4U_7-MOE**	80 h
M6	**S, MOE-T, MOE-C, U_4U_7-F**	80 h
M7	**S, MOE-T, MOE-C, U_4U_7-NH_2**	80 h

Beside improving the stability, the 2'-methoxyethoxy modifications may have an influence on the lipophilicity of the ribozyme. Increased lipophilic character should facilitate the membrane permeability. The partition coefficients between 1-octanol and water and the retention times of the nucleoside analogues reflect the change in lipophilicity. The data of the 2'-modified nucleosides in Table 4 were determined using 2'-deoxythymidine and uridine as references. Both, the partition coefficients and the retention times, indicate that the modified nucleosides are as lipophilic as 2'-deoxythymidine. The retention times obtained from reversed phase HPLC for

2'-methoxyethoxy nucleoside analogues are the highest of all analogues (Table 4). Alltogether, we synthesized highly stable ribozymes with very good catalytic activities and with a higher lipophilicity of the monomers.

Table 4. Partition coefficient and retention time for nucleosides analogues

Nucleoside	partition coefficient P	retention time t_R [min]
2'-Deoxythymidine (dT)	0.060[a]	n.d.
Uridine (U)	0.016	5.6
2'-Fluoro-2'-deoxyuridine (FU)	0.050	6.6
2'-O-Methyluridine (OMeU)	0.045	6.8
2'-O-Methoxyethoxythymidine (MOE-T)	0.053	7.5

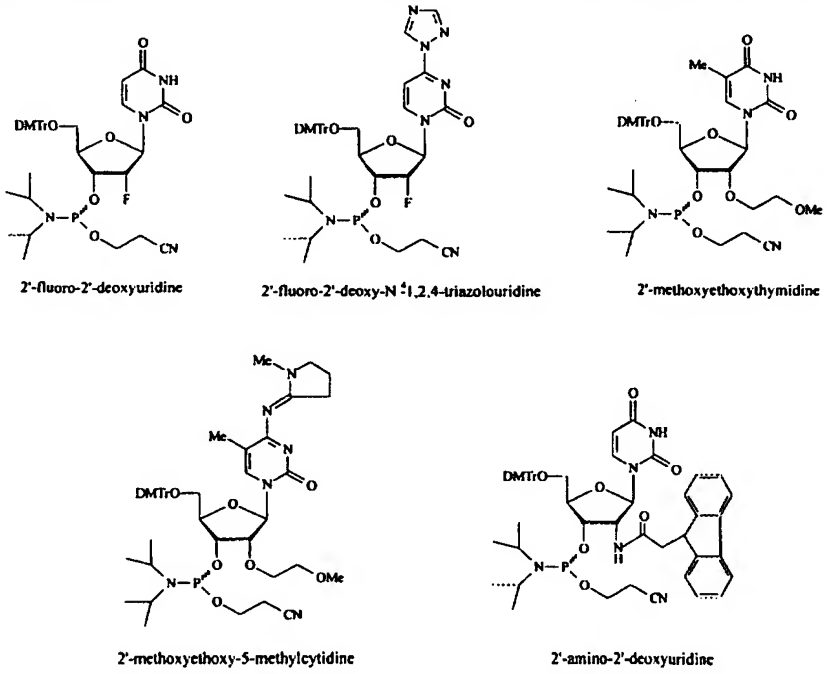
[a] The partition coefficient was taken from the paper of Lien[12]; [n.d. = not determined].

Synthesis of the modified nucleotides and oligonucleotides

The 2'-methoxyethoxy modified ribonucleosides[13] were a kind gift of Dr. P. Martin, Novartis, and the 2'-amino-2'-deoxyuridine was a kind gift of K. Jahn-Hoffmann.

Beside 2'-methoxyethoxy modifications, 2'-fluoro groups were incorporated into the ribozymes. The synthesis of 2'-fluoro-2'-deoxypyrimidine nucleosides and their incorporation into the hammerhead ribozymes together with 2'-methoxyethoxy and 2'-amino-2'-deoxy compounds was much more efficient and gave higher yields than described in Shah[14] (Figure 2). The procedure is described below.

Figure 2. Different 2'-modified ribonucleosides incorporated in oligoribonucleotides



Modified synthesis of 2'-fluoro-2'-deoxyphosphoramidite

Uridine was transformed into 2,2'-anhydro-uridine according to the procedure of Verheyden et al.[15] with HMPT. This was the starting material of the synthesis of 2'-fluoro-2'-deoxyuridine using HF-pyridine as fluorinating agent[16]. The 2'-fluoro-2'-deoxyuridine-3'-phosphoramidite was prepared by the method of Sinha[17]. The transformation of the 2'-fluoro-2'-deoxyuridine-3'-phosphoramidite to the 2'-fluoro-2'-deoxy-N^4-1,2,4-triazolouridine-3'-phosphoramidite is given below.

5'-O-dimethoxytrityl-2'-fluoro-2'-deoxy-N^4-1,2,4-triazolouridine-3'-O-(O-cyanoethyl-N,N-diisopropylamino)-phosphite

To an ice-cooled stirred suspension of 1,2,4-triazole (1.393 g, 20.2 mmol) in dry acetonitrile (26 ml) ($H_2O<10$ ppm) was added slowly $POCl_3$ (0.67 g, 4.38 mmol) followed by dry Et_3N (3 ml). After 30 min a solution of 2'-fluoro-2'-deoxyuridine-3'-phosphoramidite (250 mg, 0.33 mmol) in dry acetonitrile (6.6 ml) was added over a period of 15 min. Ice-cooled stirring was continued for 2 h followed by 1.5 h at room temperature. The reaction solution was diluted with methylenechloride (60 ml) and extracted with a saturated $NaHCO_3$ solution (60 ml) and brine (30 ml). The organic layer was dried with Na_2SO_4 and evaporated to dryness. The crude compound was purified by column chromatography (ethyl acetate:hexane 8:2) and can be directly used as cytidine analogue in the oligonucleotide synthesis. Yield: 163 mg, 62%. 1H NMR ($CDCl_3$) (ppm):9.25 (s, 1H, C-H, 1,2,4-triazole); 8.82 (d, 1H, H-6); 8.08 (s, 1H, C-H 1,2,4-triazole); 7.39-6.82 (m, 13H, arom. H); 6.54 (d, 1H, H-5); 6.25 (d, 1 H, H-1'); 5.2 (dd, 1 H, H-2'); 4.83-4.71 (m, 1H, H-3'); 4.36 (d, 1 H, H-4'); 3.81 (d, 6H, $-OCH_3$); 3.79-3.52 (m, 6H, 2CH, H-5', H-5'', OCH_2); 2.46 (m, 2 H, CH_2CN); 1.29-1.13 (m, 12 H, $-CH_3$); ^{31}P NMR ($CDCl_3$) (ppm): 152,7 (d, J_{P-F} 5,7 Hz); 151,7 (d, J_{P-F} 9,6 Hz); ESI-MS (+) 799, 32 g/mol (800,9 g/mol); TLC (toluene:ethyl acetate 1:5); RF 0.52 and 0.43 (two diastereoisomers).

The 2'-modified phosphoramidites were introduced into the ribozyme with the aid of an Applied Biosystems 380B DNA Synthesizer on a 1 μmol scale with final detritylation on the synthesizer. Oligoribonucleotides were synthesized and deprotected using standard protocols with 600 sec. coupling time for the modified phosphoramidites except for the oxidation procedure that was performed using tert.-butylhydroperoxide. The ribozymes were purified by polyacrylamide gel electrophoresis as described previously[9]. RNA concentration was measured by assuming an extinction coefficient at 260 nm of $6.6 \times 10^3\ M^{-1}\ cm^{-1}$ [18].

The partition coefficients were determined in 1-octanol and water. Retention times were measured on a LiChrospher RP 18 columm (3 mm x 125 mm) with acetonitrile/water as eluent. Nucleosides were eluated with a 20 min gradient from 0 to 100 % acetonitrile.

The cleavage kinetics with short synthetic oligoribonucleotide substrate or with the long N-*ras* transcript were performed as described previously [9]. The single-turnover k_{react}/K_M-values were determined as described previously [8].

The stability of ribozymes in cell culture supernatants supplemented with 10% FCS was performed as described previously [9]. The degree of RNA digestion was visualized by silver staining and quantified by laser scanning densitometry. Half-life times were determined by the ratio between full-length oligoribonucleotides/degradation products and full-length oligoribonucleotides.

Acknowledgment - We thank B. Conrady for supporting the reversed phase HPLC chromatography. This work was supported by DFG and Fonds der chemischen Industrie.

References

1. Koizumi, M.; Kamiya, H.; Ohtsuka, E. *Gene* **1992**, *117*, 179.
2. Scanlon, K.J.; Jiao, L.;Funato, T.; Wang, W.; Tone, T.; Rossi, J.J.; Kashani-Sabet, M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*,10591.
3. Kientopf, M.; Brach, M.A.; Licht, T., Petschauer, S., Karawajew, L.; Kirschning, C.; Herrmann, F *EMBO J.* **1994**, *13*, 4645.
4. Rossi, J.J.; Elkins, D.; Zaia, J. A.; Sullivan, S. *AIDS Res. Hum. Retroviruses* **1992**, *8*, 183.
5. Uhlenbeck, O.C. *Nature* **1987**, 328, 596.
6. Ruffner, D.E.; Stormo, G.D.; Uhlenbeck, O.C. *Biochemistry* **1990**, *29*, 10695.
7. Haseloff, J.; Gerlach, W.L. *Nature* **1988**, *334*, 585.
8. Heidenreich, O.; Benseler, F.; Fahrenholz, A.; Eckstein, F. *J. Biol. Chem.* **1994**, *269*, 2131.
9. Scherr, M.; Grez, M.; Ganser, A.; Engels, J.W. *J. Biol. Chem.* **1997**, June issue, in press.
10. Heidenreich, O.; Xu, X., Swiderski, P.; Rossi, J.J.; Nerenberg, M. *Antisense & Nucleic Acid Drug Dev.* **1996**, 6, 111-118.
11. Beigelman, L.; McSwiggen, J.A.; Draper, K.G.; Gonzalez, C.; Jensen, K.; Karpeisky, A.M.; Modak, A.S.; Matulic-Adamic, J.; DiRenzo, A.B.; Haeberli, P.; Sweedler, D.; Tracz, D.; Grimm, S.; Wincott, F.E.; Thackray, V.G.; Usman, N. *J. Biol. Chem* **1995**, 270, 25702-25708.
12. Lien, E.J.; Gao, H.; Prabhakar, H. *J. of Pharm. Sciences* **1991**, *80*, 517.
13.Martin, P.; *Helv. Chim. Acta* **1995**, *78*, 486-504.
14. Shah, K.; Wu, H.; Rana, T.M. *Bioconjugate Chem.* **1994**, *5*, 508.
15. Verheyden, J.P.H.; Wagner, D.; Moffatt, J.G. *J. Org.Chem.* **1971**, *36*, 250.
16. Yoneda, N. *Tetrahedron* **1991** *47*, 5329.
17. Sinha, N.D.; Biernat, J.; Köster, H. *Tetrahedron Letters* **1983**, *24*, 5843.
18. Fedor, M.J.; Uhlenbeck, O.C. *Biochemistry* **1992**, *31*, 12042.

(*Received in Belgium* 7 *April* 1997; *accepted* 9 *June* 1997)